Blood Flow: Metalloproteases Cut Loose in Primitive Erythrocytes

Little is known about how blood begins to flow during development. A new study shows that release of primitive blood cells in the zebrafish embryo is synchronized and mediated by a metalloprotease.

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During development, the vertebrate embryo rapidly increases in size and cannot sustain its need for oxygen by diffusion alone. A primitive wave of hematopoiesis begins just before the onset of circulation and establishes a pool of erythrocytes that will support growth of the embryo [1]. The timing of this first hematopoietic event must be tightly coordinated with organization of the vascular network and the first contractions of the primitive heart tube. Each component of the cardiovascular system supports the other as it begins to function: heart contractions create the plasma flow that distributes erythrocytes; the shear stress and viscosity of circulation help to remodel and tone the vasculature; the endothelial walls of the vessels themselves become a site of hematopoiesis [2–7]. While the interdependence of the early circulatory system is essential, the many variables make it challenging to study. Writing in this issue of *Current Biology*, Iida and colleagues [8] have used live imaging and an array of genetic tools in zebrafish to tackle the onset of circulation.

In the mouse embryo, where early hematopoietic events have been more intensely studied, the first hematopoietic cells are found in the blood islands of the extra-embryonic yolk sac [9]. These cells are predominantly primitive erythrocytes and some myeloid progenitors. With the onset of circulation (between somite stages 4 and 8) primitive erythrocytes start to redistribute throughout the embryo proper [2,3,10]. At first this redistribution is limited, partly because cardiac output is not robust, but also because a complete vascular loop has not been connected (i.e. the cardinal vein has not formed) [10]. However, as blood flow increases over the next few hours, thousands of erythrocytes become distributed throughout the embryonic vasculature. Interestingly, steady-state levels of red blood cells are not reached until embryonic day 10, two full days after circulation begins. This suggests that the release of erythrocytes from the yolk sac blood islands into the embryo proper must be tightly regulated to match cardiac output and vascular maturity.

The redistribution of blood cells in the embryo requires circulation, although little else is known about how release of these cells is regulated. One observation from the initiation of erythrocyte circulation in the mouse yolk sac was that some cells circulate freely while others clump together and become stuck in the vascular network [3] — suggesting a change of adhesive properties in primitive blood cells as they are released. Another observation was that increasing viscosity of the plasma, associated with release of more erythrocytes from the yolk sac, is required for remodeling of the yolk sac vasculature [3]. Increasing viscosity creates biomechanical feedback, ultimately resulting in release of more blood cells into the circulation. Furthermore, shear stress within the vasculature actually drives expansion and development of hematopoietic progenitors [11,12]. Together, these observations indicate that multiple mechanisms work together to establish robust circulation in the developing embryo.

The zebrafish is a unique model system to study hematopoiesis because its blood cell types are highly comparable to mammals, there is an array of available genetic tools and an optically clear embryo allows *in vivo* tracking of development [1]. In zebrafish, primitive erythrocytes form within the intermediate cell mass, which lies in the trunk of the embryo, closely apposed to the dorsal aorta and posterior cardinal vein (Figure 1). The onset of circulation occurs between 23 and 26 hours post-fertilization: the heart begins to generate plasma flow, the cardinal vein forms and closes a circulatory loop and blood cells are released into circulation. As these events occur at approximately the same time, it is unclear if release of blood cells is initiated by plasma flow alone, or if there is an active mechanism that regulates their release.

To track the beginning of blood flow in the embryo, Iida and colleagues [8] combined two transgenic lines in the zebrafish that mark primitive erythrocytes and blood vessels (*gata1*:mRFP [13,14] and *fli1a*:eGFP [15], respectively). Imaging of the double transgenic revealed that before circulation primitive blood cells were largely segregated into a sub-aortic compartment (Figure 1A). Even after the initiation of plasma flow, these primitive erythrocytes did not enter circulation, but instead were observed migrating into the dorsal aorta and posterior cardinal vein — a process the authors termed ‘intravasation’ (Figure 1B). Once in the lumen of the vessel, erythrocytes remain attached to the endothelial wall (Figure 1C). Shortly afterwards, there was a dramatic and almost simultaneous release of virtually all primitive erythrocytes into circulation (Figure 1D). This key observation led Iida and colleagues [8] to explore a possible mechanism for the coordinated release of primitive blood cells.

The primary hypothesis of Iida and colleagues [8] was that blood cells attached to the endothelial wall by extracellular adhesion molecules could be released by proteolytic degradation. A close association between blood and endothelial cells, both during intravasation and before the onset of circulation, was confirmed using high-powered imaging and electron microscopy. Expression of the focal-adhesion protein vinculin was also observed between blood and endothelial cells while they were attached, and was then absent after the initiation of blood flow. Metalloproteases were considered

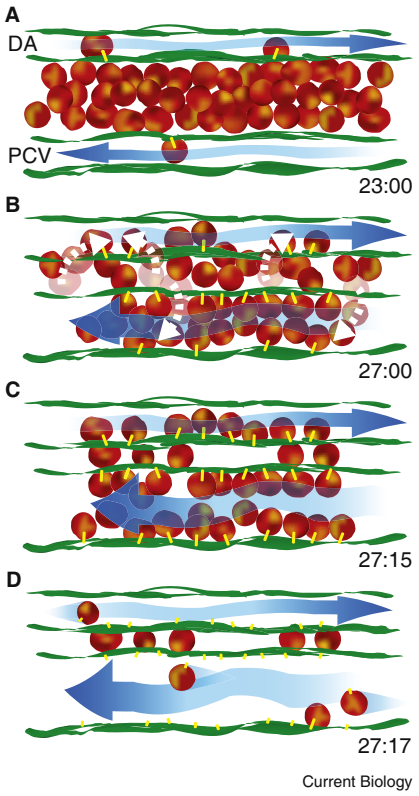

Figure 1. Onset of blood flow in the zebrafish embryo.

(A) When the heart starts beating at approximately 23 hours post fertilization, the majority of primitive blood cells are in the sub-aortic space between the dorsal aorta (DA) and the posterior cardinal vein (PCV). The blood cells (red) have not entered the plasma flow (blue arrows). (B) Over the next few hours (times are given in the bottom right of each panel), blood cells undergo a process of intravasation; they move into the lumen of the vessels and remain attached to the endothelial wall (green). (C) Once intravasation is complete the blood cells idle in the vessels. (D) Suddenly, blood cells are released synchronously into circulation. This step requires a metalloprotease that cleaves the adhesion molecules (yellow bars) holding blood cells to the vessel walls. Without metalloprotease function, cells remain stuck in the idling phase (C).

as candidates for the proteolytic processing of attachments between blood and endothelial cells. Injection of metalloprotease inhibitor directly into the plasma flow, together with a labeled dextran marker to confirm continued circulation, resulted in stagnation of blood cells. A search for blood-specific metalloproteases revealed *adam8* as a primary candidate. Strikingly, morpholino knockdown of *adam8* in the developing embryo resulted in significant stagnation of blood cells, without disruption of vascular formation, heart function, or plasma flow. The endogenous expression pattern of *adam8* was observed in blood but not endothelial cells, suggesting a possible cell-autonomous role for the metalloprotease. To address this, metalloprotease-inactive *adam8* was expressed specifically in erythrocytes, resulting in a dominant negative inhibition of blood cell release — a result that strongly supports a cell-autonomous role for adam8 in proteolysis of blood-vessel adhesions.

Taking advantage of the zebrafish, Iida and colleagues [8] have shown that primitive blood cells undergo synchronized and active release into the circulation. The biological significance of this event is still unclear. It may be that some blood cells are released when they reach a specific differentiation state; others may be retained until they differentiate further. Synchronous release may also prevent blood cells from entering circulation before the vascular system can provide continuous circulation. An important outstanding question is how synchronous release is actually coordinated. The authors propose one model that would require sufficient maturation of the vessel wall, which then transfers a signal to the blood cells that activates metalloproteases and triggers release into circulation. In another model, proteolysis of blood-vessel adhesions is a prerequisite for their release, but not the trigger; release would require another mechanism, such as a threshold of plasma flow or removal of an additional, inhibitory molecule.

It will be intriguing to explore the role of proteolytic release of blood cells in mammalian embryos. Although the release of primitive erythrocytes in the mouse embryo is more gradual than in zebrafish, the vascular network also matures over a much longer period of time [10]. Perhaps metalloproteases also play a role in the mouse yolk sac, but are triggered by increasing flow as the yolk sac vasculature is remodeled. The question of how blood cells are released into circulation will undoubtedly be unraveled using the increasing number of genetic tools and live imaging possibilities in both mouse and zebrafish.

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DOI: 10.1016/j.cub.2010.05.014